CHAPTER 3

RESULT

3.1 Subcloning of DNA fragment from pCSBC14

Tanunat (1995) had constructed pCSBC14 which was a pUC18 recombinant plasmid, comprising of a 4.0 kb chromosomal DNA fragment of *B. subtilis* TISTR25. This protease activity and restriction mapping of the DNA insert were reported. Study will be further conducted to confirm whether it consisted of protease gene or not by DNA sequencing.

Approximately 1 μg of extracted pCSBC14 DNA was digested with both *Hin*dIII and *Eco*RI, resulting in two *Hin*dIII/*Eco*RI fragments (0.7 and 0.9 kb) and one *Hin*dIII/*Hin*dIII fragment (1.6 kb) of the 4.0 kb insert fragment (Figure 3.1). Two smaller fragments, 0.2 kb *Hin*dIII/*Hin*dIII and 0.1 kb *Hin*dIII/*Eco*RI fragments, were not seen in Figure 3.1. These 3 fragments were separated from each other by 0.7% agarose gel electrophoresis and eluted with QIAEX II Agarose Gel Extraction Kit. The yield of DNA was estimated to be 80% recovery. Then, each of the 0.7 and 0.9 kb fragment was ligated into *Hin*dIII and *Eco*RI sites of M13mp18. The 1.6 kb fragment was ligated into *Hin*dIII sites of M13mp18 (Figure 3.1).

The 3 recombinant clones, comprising of 0.7, 0.9 and 1.6 kb insert fragments were named as mCSBC141, mCSBC142 and mCSBC143, respectively. Each of the 3 clones was constructed by subcloning of the fragment into M13mp18. The ligation mixtures were transformed into *E. coli* JM109 by electroporation and grown on LB plate with X-gal and IPTG. The ratio of white plaques to blue plaques of the cloning of mCSBC141, mCSBC142 and mCSBC143 were 102:54, 108:61 and 15:20, respectively. The efficiencies of transformation were 1.66×10^5, 1.69×10^5 and 3.5×10^4 plaques/μg DNA, respectively.

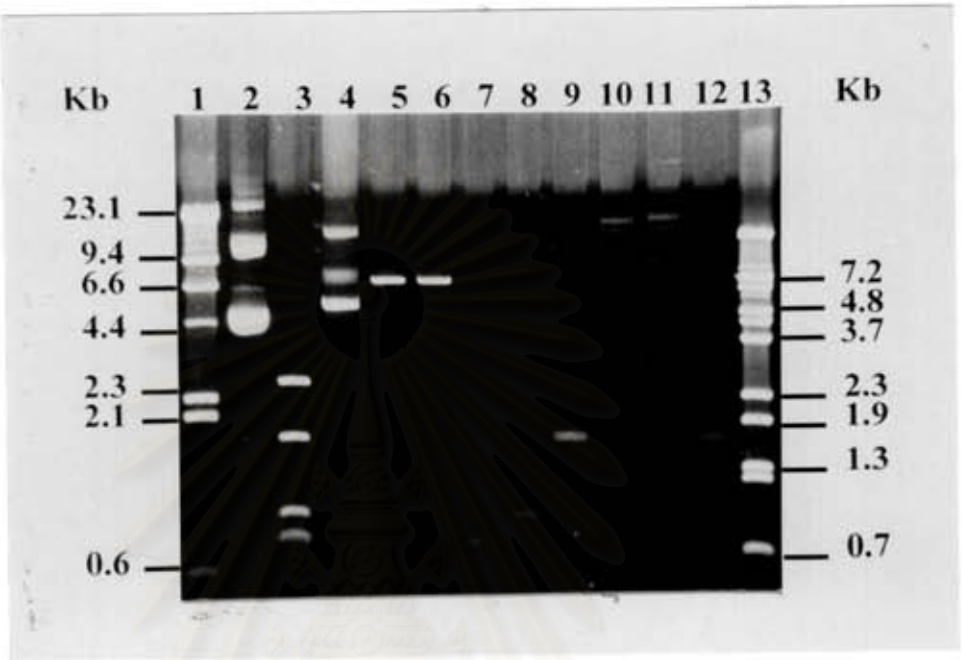


Figure 3.1 Ligation products of 3 DNA fragments, 0.7, 0.9 and 1.6 kb, from *Hin*dIII and *Eco*RI digestion of pCSBC14 into M13mp18

Lane 1	λ/*Hin*dIII
Lane 2	Uncut pCSBC14
Lane 3	*Hin*dIII and *Eco*RI digested pCSBC14
Lane 4	Uncut M13mp18
Lane 5	*Hin*dIII digested M13mp18
Lane 6	*Hin*dIII and *Eco*RI digested M13mp18
Lane 7	Isolated 0.7 kb DNA fragment
Lane 8	Isolated 0.9 kb DNA fragment
Lane 9	Isolated 1.6 kb DNA fragment
Lane 10	Ligation product of 0.7 kb fragment
Lane 11	Ligation product of 0.9 kb fragment
Lane 12	Ligation product of 1.6 kb fragment
Lane 13	λ/*Bst*EII

The RF forms of the 3 recombinant M13 clones were extracted by alkaline extraction, described in section 2.8.5.1 and then digested with restriction endonucleases in order to determine the corrected insert fragments. The mCSBC141 and mCSBC142 were digested with both *Hin*dIII and *Eco*RI, and the mCSBC143 was digested with only *Hin*dIII. The insert fragment size of mCSBC141, mCSBC142 and mCSBC143 were determined as 0.7, 0.9 and 1.6 kb, respectively (Figure 3.2).

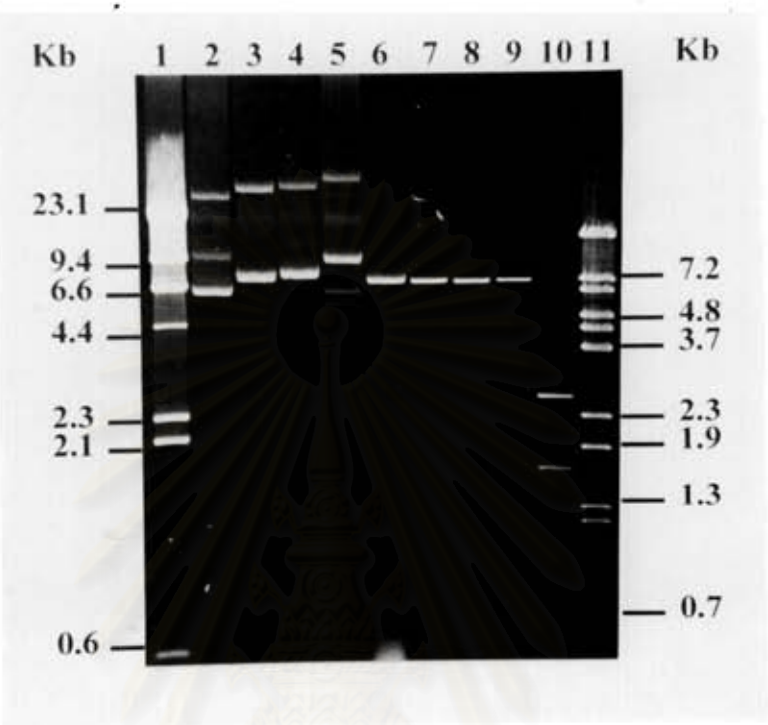


Figure 3.2 Determination of the insert fragments of 3 transformants

Lane 1	λ/*Hin*dIII
Lane 2	Uncut M13mp18
Lane 3	Uncut mCSBC141
Lane 4	Uncut mCSBC142
Lane 5	Uncut mCSBC143
Lane 6	*Hin*dIII digested M13mp18
Lane 7	*Hin*dIII and *Eco*RI digested mCSBC141
Lane 8	*Hin*dIII and *Eco*RI digested mCSBC142
Lane 9	*Hin*dIII digested mCSBC143
Lane 10	*Hin*dIII and *Eco*RI digested pCSBC14
Lane 11	λ/*Bst*EII

3.2 DNA sequencing

3.2.1 Sequencing of pCSBC14

The pCSBC14 was extracted by alkaline extraction as described in section 2.6. The DNA was sequenced on both sides of the 4.0 kb insert fragment with 17-mer forward primer (5′-TGACCGGCAGCAAAATG-3′) and 24-mer reverse primer (5′-AGCGGATAACAATTTCACACAGGA-3′) by automated DNA sequencer (Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus). The results were showed in Figure 3.3 and 3.4. The nucleotide sequences of 600 bp at the 5′end and 610 at 3′end were determined.

3.2.2 Sequencing of mCSBC141, mCSBC142 and mCSBC143

The 0.7, 0.9 and 1.6 kb insert fragments of mCSBC141, mCSBC142 and mCSBC143, respectively, were sequenced by manual method described in section 2.9.2.2. The mCSBC141 and mCSBC142 were extracted as RF form and single-stranded DNA. The RF form and single-stranded DNA were sequenced with 24-mer reverse primer (5′-AGCGGATAACAATTTCACACAGGA-3′) and 17-mer forward primer (5′-CAGCACTGACCCTTTTG-3′), respectively. The mCSBC143 was extracted as single-stranded DNA and sequenced in one direction with forward primer.

The 0.7 kb insert fragment of mCSBC141 was sequenced in both directions which revealed about 445 bp at the 5′end (Figure 3.5a, b) and 426 bp at the 3′end (Figure 3.6a, b).

The 0.9 kb DNA insert of mCSBC142 was also sequenced in both directions. The nucleotide sequence of about 433 bp at the 5′end (Figure 3.7a, b) and 551 bp at the 3′end (Figure 3.8a, b) were determined.

Unlike mCSBC141 and mCSBC142, mCSBC143 was sequenced only with forward primer. The result showed about 302 bp at the 5′end of the 1.6 kb insert fragment (Figure 3.9a, b).

The remaining about 700 bases in the middle of the insert in mCSBC143 was not sequenced.

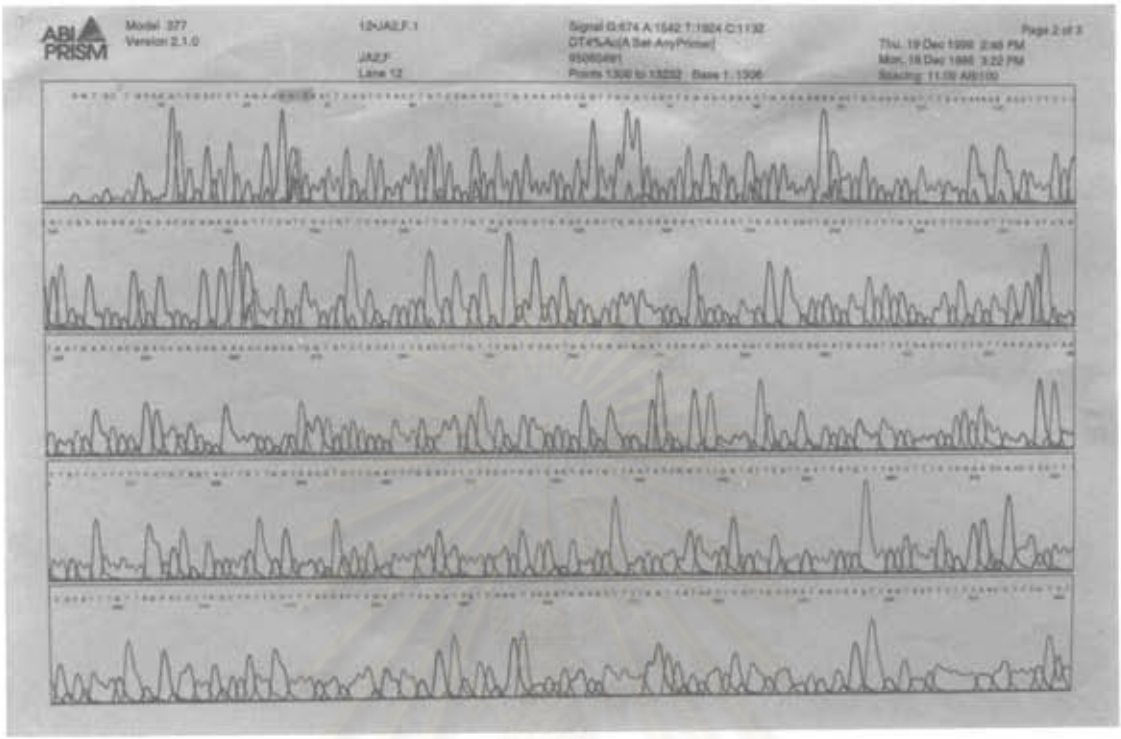


Figure 3.3 Automated DNA sequencing of pCSBC14 with forward primer

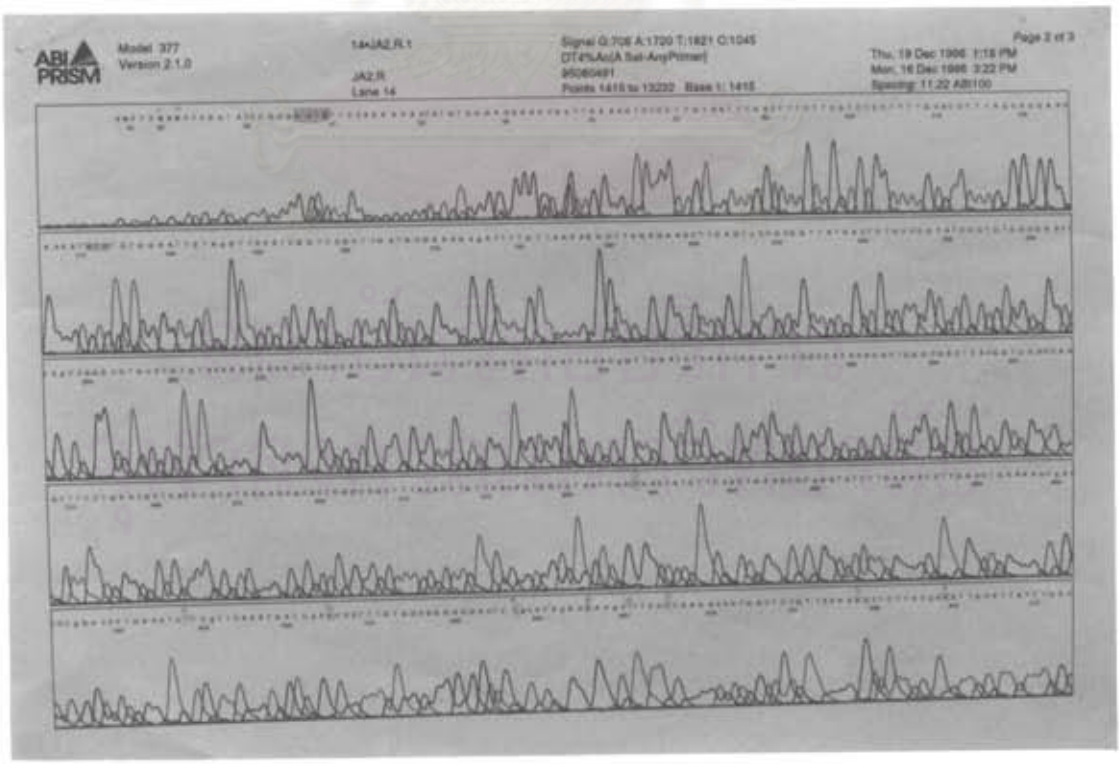
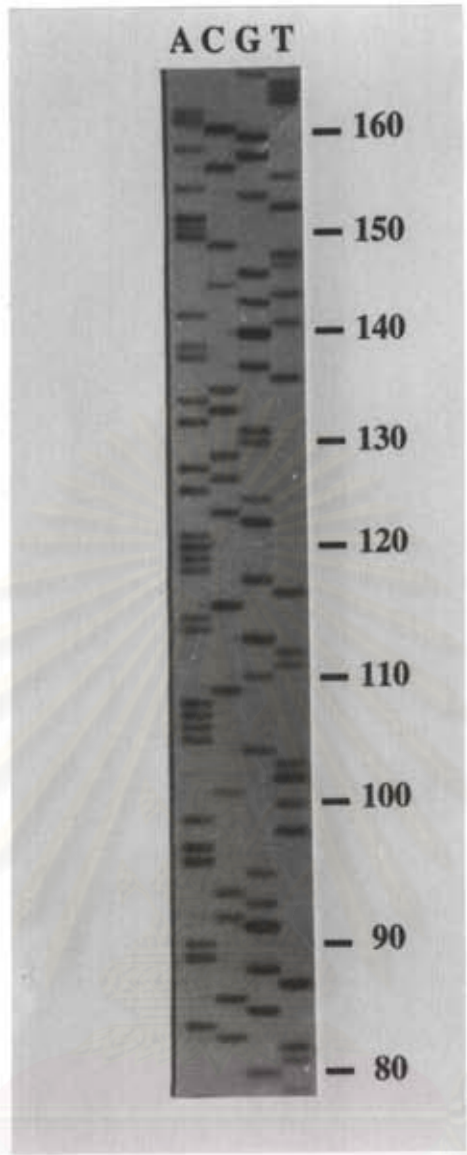


Figure 3.4 Automated DNA sequencing of pCSBC14 with reverse primer



(a)

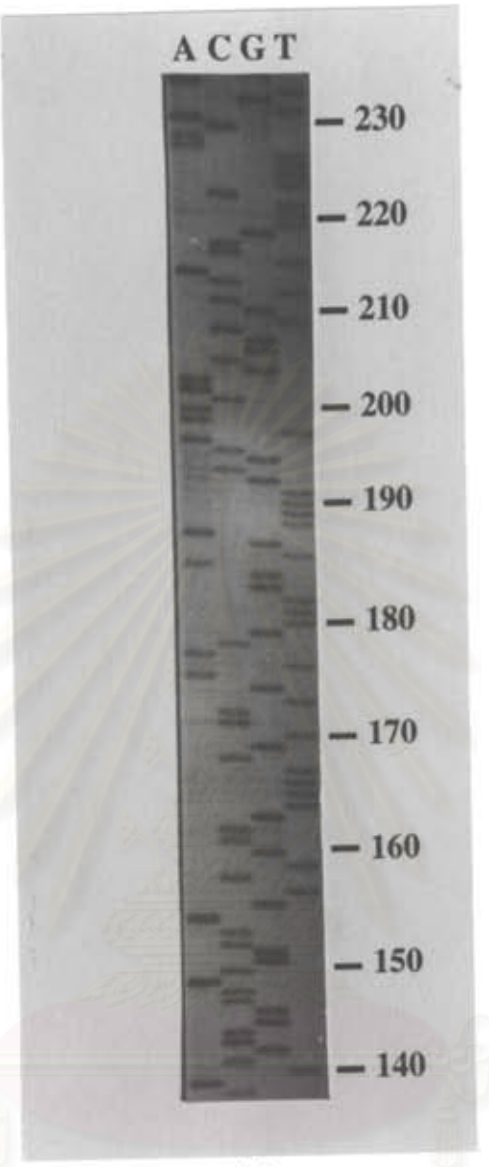
```
  1 AAGCTTCCTG AACGCTCACC CGCATCAAAG CGCACTCGGC CGCTTTACAC TTGTTCACAA
 61 TGGTGTGATC GAGAACTATG TTCAGCTGAA GCGCGAATAT CTTGAAAACG TTGAACTGAA
121 AAGCGACACG GACACTGAAG TAGTCGTTCA AATGATCGAG CAATTTGTGG CGGGAGGACT
181 CAGCACAGAA GAAGCGTTCC GCAAAACACT GACTCTGTTA AAAGGCTCTT ACGCAATTGC
241 ATTATTTGAC GGTGAAAACA CAGACACCAT TTACGTTGCA AAAAACAAAA GCCCTCTGTT
301 AATCGGCCTT GGAGATACGT TTAACGTCGT GGCATCTGAC GCGATGGCTA TGCTTCAAGT
361 AACGAATGAA TACGTTGAGC TTTTGGACAA AGAAATGGTG ATCGTGACAA AAGATGAAGC
421 CGTGATTAAA AACCTTGACG GTGAA
```

(b)

Figure 3.5 Sequencing of mCSBC141 with forward primer (5' side of the 0.7 kb insert)

(a) Autoradiograph of 6% polyacrylamide gel, lane A = ddATP, lane C = ddCTP, lane G = ddGTP and lane T = ddTTP

(b) Nucleotide sequence of 445 bases



(a)

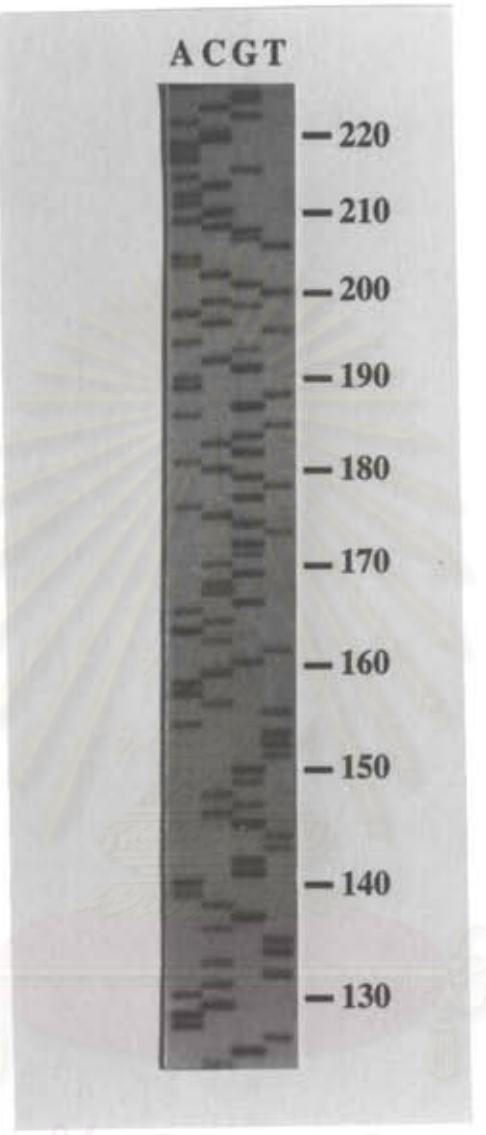
```
  1 GAATTCACTC GCTACATGCA CTTCAACCGG TACGTTTGCC CACATTTCAA TATATTGTTT
 61 CCCGACAAGA CCGGCGTGGT AGCTCGTTCC GCAAGCCACG ATATAAATGC GGTCCGCTTC
121 CGCCACGGCG TCAGCGACAT CGCCGGCCAC GGCCAGTCTG CCGTTTTCGT CCTGATACGT
181 TTGGATGATT TTGCGCATAA CAAGCGGCTG CTCATCCGTT TCTTTTAACA TGTAGTGAGG
241 GTATGTGCCT TTTTCGATAT CACTGGCGTC AAGCTCAGCG ATATAAGACG CACGTGTCAT
301 GACTTCACCG TCAAGGTTTT TAATCACGGC TTCATCTTTT GTCACGATCA CCATTTCTTT
361 GTCCAAAAGC TCAACGTATT CATTCGTTAC TTGAAGCATA GCCATCGCGT CAGATGCCAC
421 GACGTT
```

(b)

Figure 3.6 Sequencing of mCSBC141 with reverse primer (3' side of the 0.7 kb insert)

(a) Autoradiograph of 6% polyacrylamide gel, lane A = ddATP, lane C = ddCTP, lane G = ddGTP and lane T = ddTTP

(b) Nucleotide sequence of 426 bases



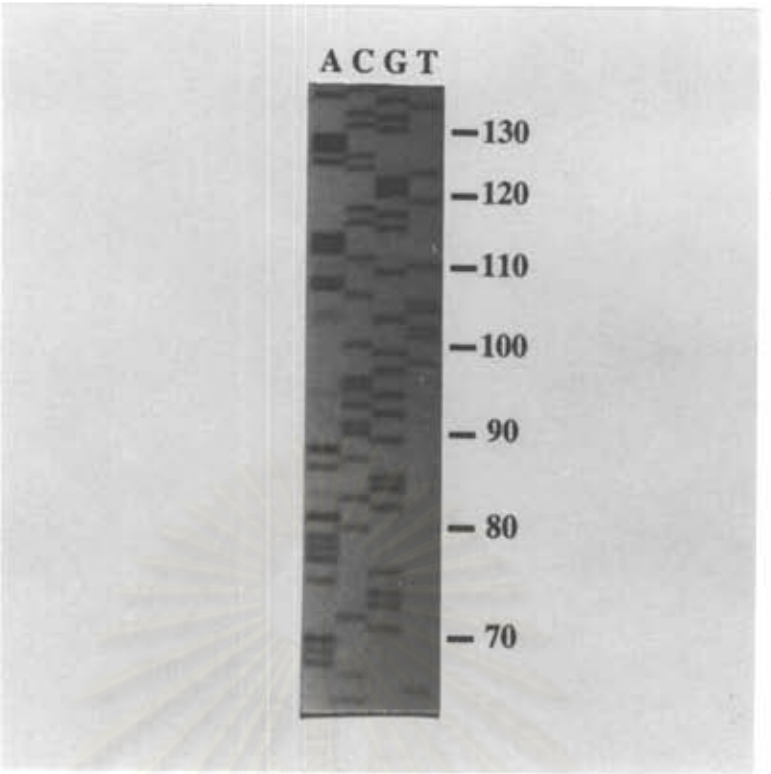
(a)

```
  1 AAGCTTCGCA ACTTTTTTAT AAACNTCGCG ACTTTATTTT AAACATCGCA TCTTTTTTAT
 61 AAACATCGCG ACTTTATTTT AAATTCACAC TAATAACCAA AGGGGTTAAA TATATTATTC
121 CACCGTAACA CTCTTCGCAA GGTTGCGCGG TTTATCA.CG TCACAGCCGC GGTGCAGTGC
181 AGCGTAGTAA GCGATCAGCT GCAATGGCAC AACAGAAACC AGCGGACGAA GCGCAGGGTT
241 GACTTCCGGC AGGATGAATC TGTCGTCTGC GTCTTCTAAG CCTTTCAGCG AGATGATGCA
301 AGTGTTGGCG CCGCGGGCTG CGACTTCCTT CACATTACCG CGGATGCTCA GGTTGACGTG
361 TTCTTGTGTC GCAAGCGCAA AGACCGGTGT TCCTTCTTCA ATCAGAGCGA TTGTTCCATG
421 CTTCAGCTCG CCG
```

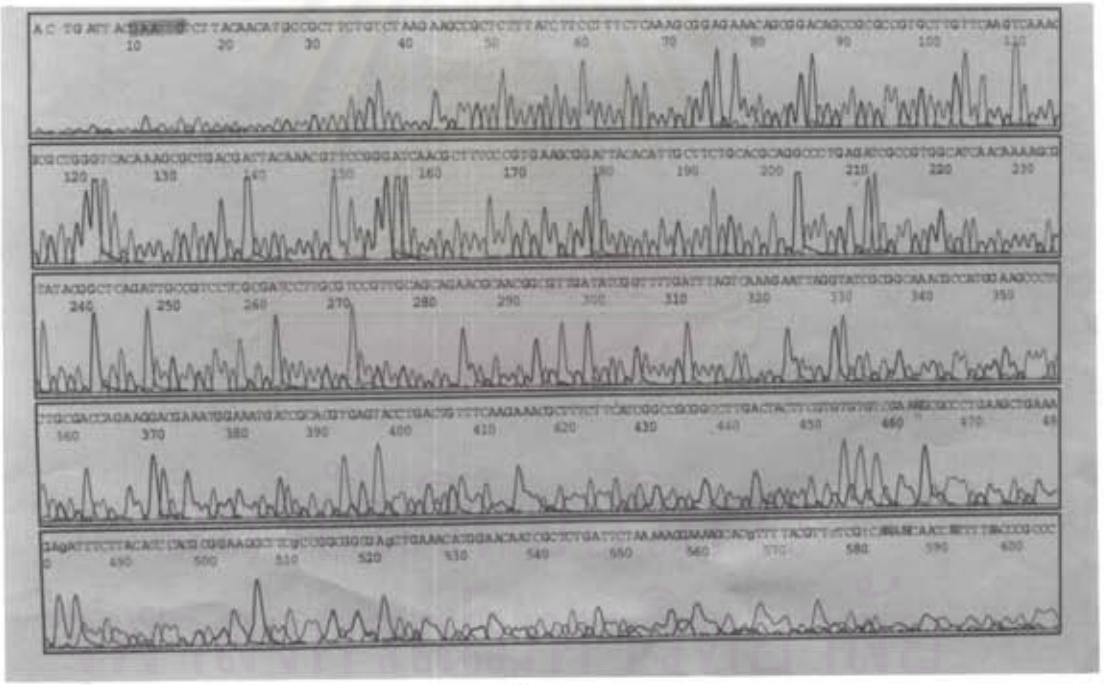
Figure 3.7 Sequencing of mCSBC142 with forward primer (5' side of the 0.9 kb insert)

(a) Autoradiograph of 6% polyacrylamide gel, lane A = ddATP, lane C = ddCTP, lane G = ddGTP and lane T = ddTTP

(b) Nucleotide sequence of 433 bases



(a)

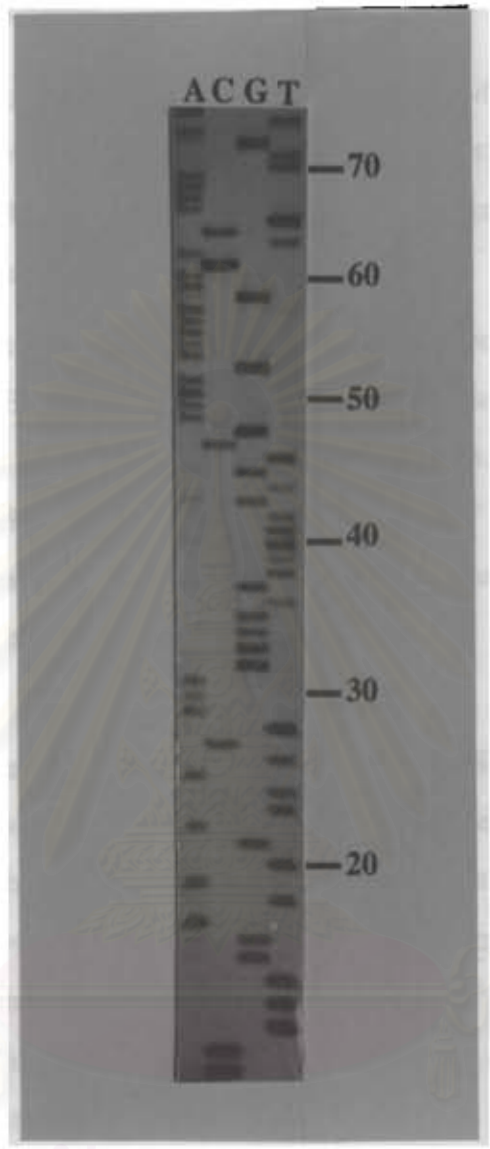


(b)

Figure 3.8 Sequencing of mCSBC142 with reverse primer (3′ side of the 0.9 kb insert)

a) Autoradiograph of 6% polyacrylamide gel, lane A = ddATP, lane C = ddCTP, lane G = ddGTP and lane T = ddTTP

b) Automated DNA sequencing of mCSBC142



(a)

```
  1 AAGCTTACCC CTTTGGATAT GATTATCTAA AGGGGTGTTT TTGTGTCGAA AAGAAAAAGA
 61 ACATCTAAAA TTGATAAGTG GATTAAAGAG GGTCGAGGAA CTGGCAGTGG GGCATGATTA
121 TCAAGATCTT TCCTCATTAG GTCGGTCAAC AAGATTAAAA GGTATATAAA CCGGCAGACA
181 ACATGAGTTT TTATCGGATT TGGAACGAAA CTACTTTTAT TTAACTGAAT TTTCTGATGT
241 TATTTTAGAT ATTCGTGAAC AATTTCCTTT ATTACCACAA GAAGAGACGT TTGCCATTGC
301 TG
```

(b)

Figure 3.9 Sequencing of mCSBC143 with forward primer (5' side of the 1.6 kb insert)

(a) Autoradiograph of 6% polyacrylamide gel, lane A = ddATP, lane C = ddCTP, lane G = ddGTP and lane T = ddTTP

(b) Nucleotide sequence of 302 bases

3.3 DNA sequence analysis

The nucleotide sequences were denoted 5′ and 3′ when forward primer and reverse primer, respectively, were used to determine the sequences. Therefore, the direction of the 5′ and 3′ sequences of mCSBC141, for example, were opposite to those of pCSBC14 and mCSBC142 (Figure 3.10).

The nucleotide sequences of pCSBC14 from both directions of the insert were compared with the GenBank deposited DNA sequences using BLAST (Basic Local Alignment Search Tool) at the website http://www.ncbi.nlm.nih.gov (Altschul *et al.*, 1990; Altschul *et al.*, 1997). The results of comparing the 5′ sequence showed that it was not significantly similar to any genes (Appendix 3). Most of the 3′ sequence was homologous to part of *B. subtilis* 168 L-glutamine D-fructose-6-phosphate amidotransferase (*gcaA*) gene (data not shown).

The nucleotide sequences of insert fragments of mCSBC141, mCSBC142 and mCSBC143 were also compared with the GenBank deposited DNA sequences. The results of comparison showed that each of the clone was similar to *B. subtilis* 168 *gcaA* gene in various positions. The *B. subtilis* TISTR25 gene started at the 3′end of pCSBC14. Therefore, the 3′ sequence of mCSBC141 and 5′ sequence of mCSBC142 were inverted to complementary strand. A total of 2,308 bp of 4.0 kb insert fragment of pCSBC14 were determined.

Analysis of the nucleotide sequence revealed an open reading frame of 1,803 bp, capable of encoding a protein of 600 amino acids (Figure 3.11). The sequences of *B. subtilis* TISTR25 gene, upstream sequence (95 bp) and downstream sequence (410 bp) were subjected to BLAST comparison. The results showed 85%, 92% and 86% identity to *B. subtilis* 168 *gcaA* gene, respectively (Figure 3.12). It was certain that the *B. subtilis* TISTR25 gene was L-glutamine D-fructose-6-phosphate amidotransferase (*gcaA*) gene. The BLAST result of 1,803 bp of *B. subtilis* TISTR25 *gcaA* gene was shown in Appendix 4. The sequences of *B. subtilis* TISTR25 and *B. subtilis* 168 *gcaA* gene were aligned by ClustalX (1.64b) as shown in Figure 3.13. From the sequence in Figure 3.11, two possible ribosomal binding sequence (Shine-

Dalgarno sequence) were AGGAGG and AGGAAG. The putative promoter regions (-10 and -35) were TTGACT and TTGAAA, respectively. The putative transcription start site was G (+1). The potential ρ-independent transcription terminator containing an inverted repeat, ACCCCTTT and AAAGGGGT, followed by a tract of T was shown. The restriction map was created by using DNA strider 1.2 program and shown in Appendix 5.

The amino acid composition was determined from the deduced amino acid sequences and compared with *B. subtilis* 168 *gcaA* protein as presented in Table 3.1. The molecular weight of *B. subtilis* TISTR25 *gcaA* protein was calculated to be 65,431 daltons. Furthermore, the predicted amino acid sequence of *gcaA* gene of *B. subtilis* TISTR25 was aligned with the *gcaA* protein of *B. subtilis* 168, *Rhizobium meliloti* (Baev *et al.*, 1991), *E. coli* (Walker *et al.*, 1984), *Candida albicans* (Smith *et al.*, 1996), *Saccharomyces cerevisiae* (Watzele and Tanner, 1989), human (McKnight *et al.*, 1992) and mouse (Sayeski *et al.*, 1994; Sayeski *et al.*, 1997) using the ClustalX (1.64b) (Figure 3.14).

The genetic distances among nucleotide sequences of 8 organisms were calculated using Kimura 2-parameter in Phylip 3.5c. The range of genetic distances was between 0.0972-1.3233 (Table 3.2). These values were used to construct phylogenetic tree (Figure 3.15). The 8 organisms can be divided into two main groups, prokaryote (*B. subtilis* TISTR25, *B. subtilis* 168, *E. coli* and *R. meliloti*) and eukaryote (*S. cerevisiae*, *C. albicans*, human and mouse). The estimated sequence divergence between the two groups was 0.33088. The estimated sequence divergence between the *gcaA* gene of *B. subtilis* TISTR25 and *B. subtilis* 168 was 0.1705 which was a lot less than that between the group of *B. subtilis* and *E. coli* (0.8801). Furthermore, eukaryote can be divided into two group, higher eukaryote (human and mouse) and lower eukaryote (*S. cerevisiae* and *C. albicans*). The estimated sequence divergence between the two groups was 0.4344. The estimated sequence divergence between the *gcaA* gene of human and mouse was 0.0972 which was less than that between *C. albicans* and *S. cerevisiae* (0.3666).

The genetic distance among amino acid sequences of 8 organisms was calculated using Dayhoff PAM matrix in Phylip 3.5c. The range of genetic distances was between 0.01002-1.38757 (Table 3.3). These values were used to construct phylogenetic tree (Figure 3.16). Two groups, prokaryote and eukaryote, were also clearly seperated. The estimated sequence divergence between the two groups was 0.4355. The estimated sequence divergence between the *gcaA* protein of *B. subtilis* TISTR25 and *B. subtilis* 168 was 0.0694. Furthermore, the estimated sequence divergence between the higher eukaryote and lower eukaryote was 0.4618. The estimated sequence divergence between the *gcaA* protein of human and mouse was 0.01002 which was less than that between *C. albicans* and *S. cerevisiae* (0.33108).

The predicted three-dimensional structure of the N-terminal domain of *B. subtilis* TISTR25 *gcaA* protein was created by using Swiss-Model at the website http://www.expasy.ch/Swissmod/SWISS-MODEL.html, Swiss-PdbViewer and Rasmol program. The 3D structure was compared with *E. coli gcaA* protein in chain A (Figure 3.17). Both structures were similar. They consisted of two layers of antiparallel β pleated sheets in the middle and sandwiched by α helices.

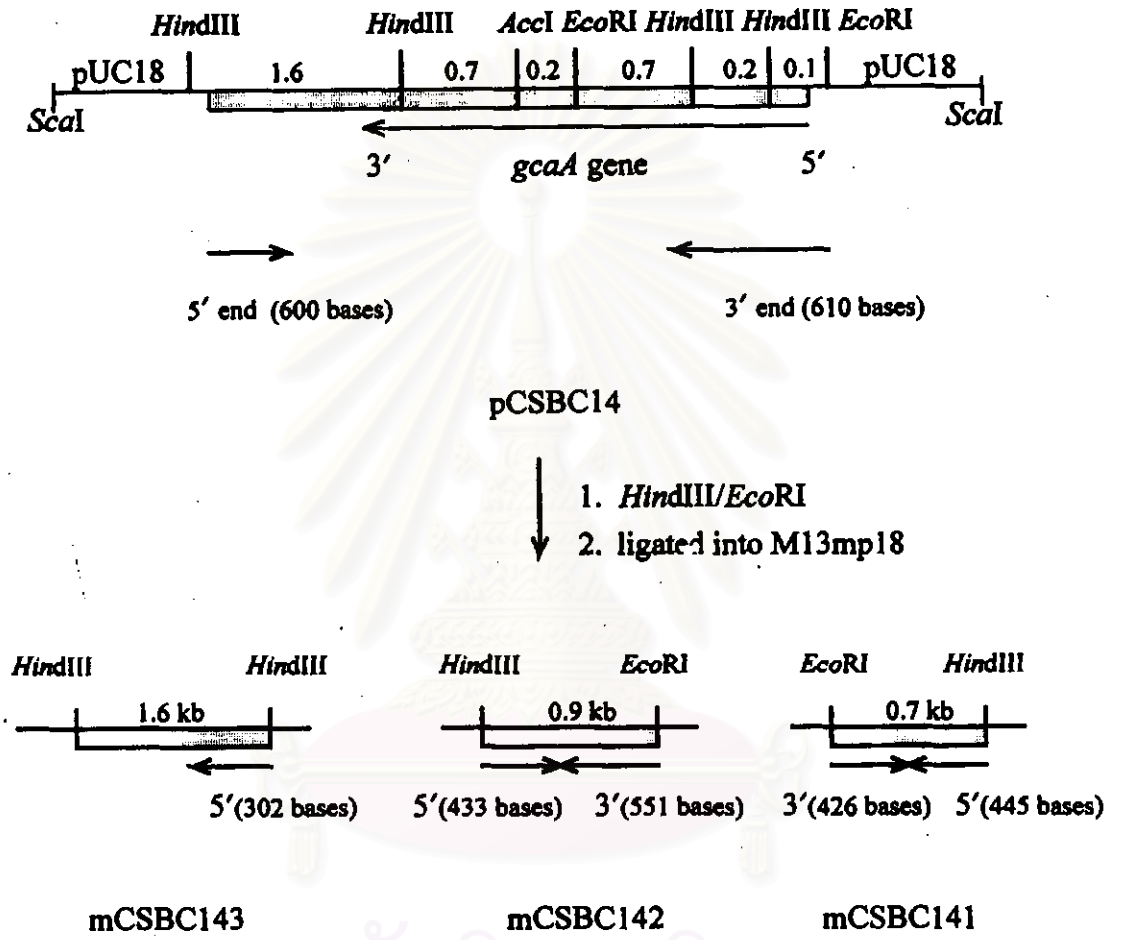


Figure 3.10 Sequencing orientation of individual clones

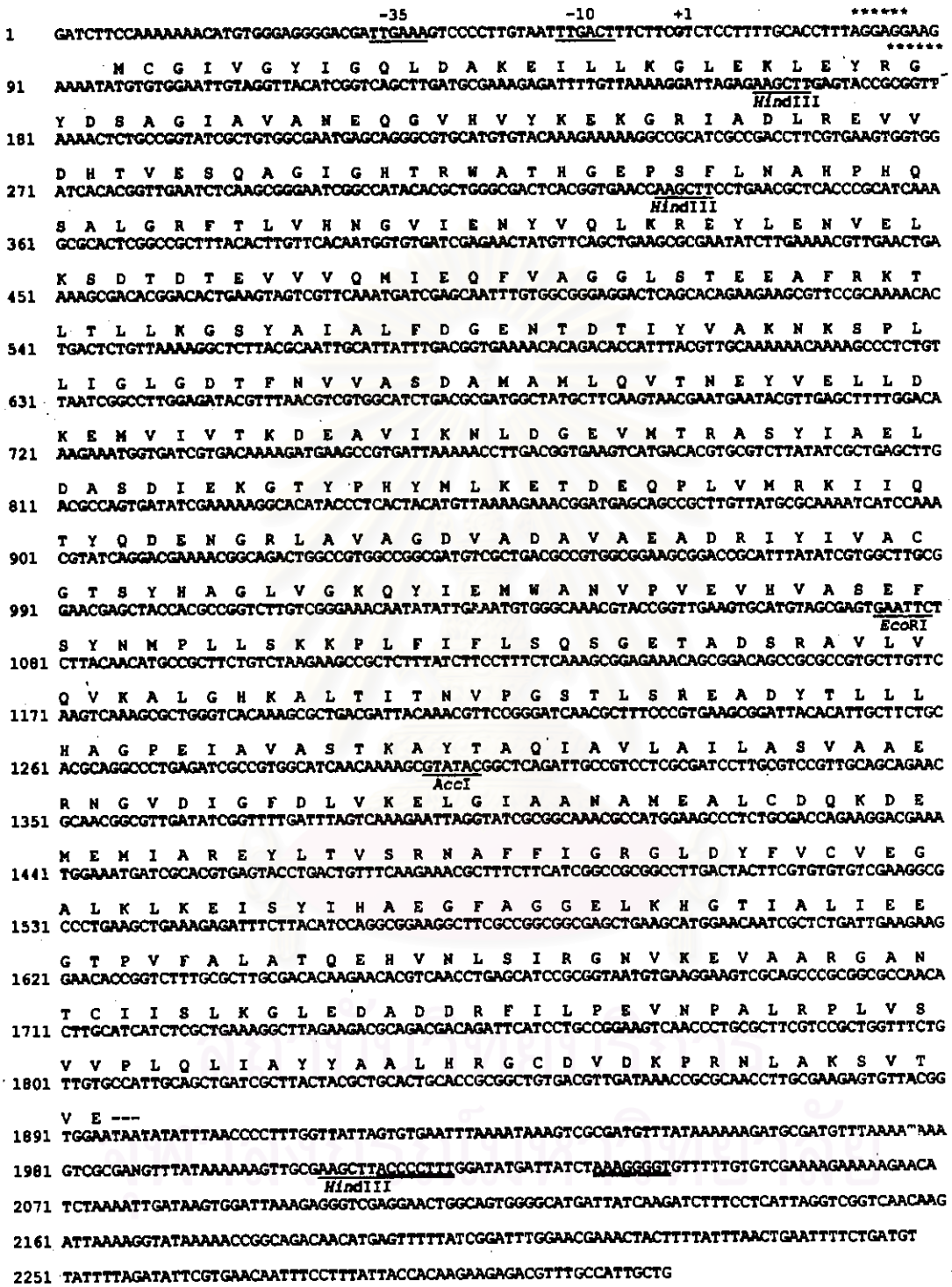


Figure 3.11 Nucleotide sequence and deduced amino acid sequence of *B. subtilis* TISTR25 *gcaA* gene. Two possible Shine-Dalgarno sequence (****) and a possible promoter (-35 and -10 regions) were indicated. The putative ρ-independent transcriptional terminator was double underlined. Termination codon (---) was indicated.

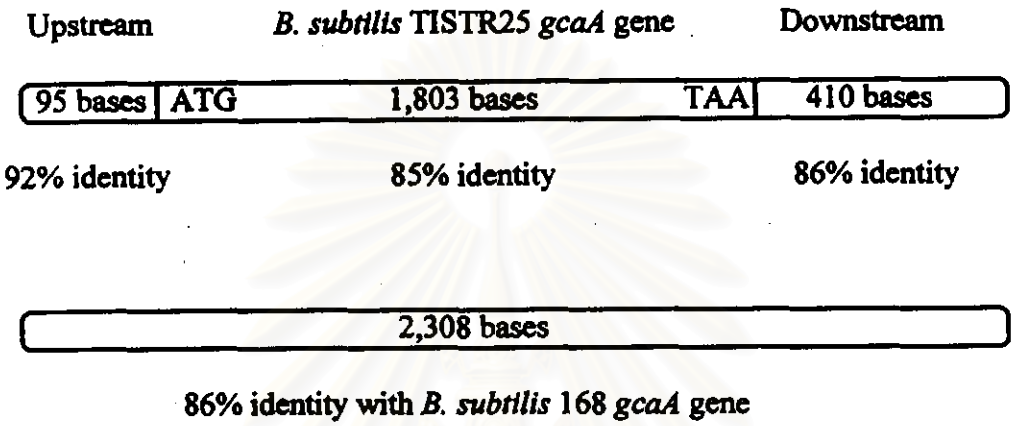


Figure 3.12 Percent identity of *B. subtilis* TISTR25 and *B. subtilis* 168 *gcaA* gene by BLAST comparison.

```
B.subtilis TISTR25      ------------------------------------------------------------
B.subtilis 168          AAACAAGCTGTCTGTTCTTATTTCTCAATAGGAAAGAAGACGGATTATTCCTTACCTATA

B.subtilis TISTR25      ------------------------------------------------------------
B.subtilis 168          ATTATAGCGCCCGAACTAAGCGCCCGGAAAAAGGCTTAGTTGACGAGGATGGAGGTTATC

B.subtilis TISTR25      ------------------------------------------------------------
B.subtilis 168          GAATTTCGCGGATCCTCCCGGCTGAGTGTGCAGATCACAGCCGTAAGAATTTCTTCAAAC

B.subtilis TISTR25      ------------------------------------GATCTTCCAAAAAAACATGTGGGA
B.subtilis 168          CAAGGGGGTGACTCCTTGAACAAAGAGAAATCACATGATCTTCCAAAAAA-CATGTAGGA
                                                            ********** ***** ***

B.subtilis TISTR25      GGGGACGATTGAAAGTCCCCTTGTAATTTGACTTTCTTCGTCTCCTTTTGCACCTTTAGG
B.subtilis 168          GGGGACGATTGAAAGTCCCCTTGAAATTTGACTTTCTTCGTCTCCTTTTACAATCTTAGG
                        ********************** ************************** **   *****

B.subtilis TISTR25      AGGAAGAAAA-TATGTGTGGAATTGTAGGTTACATCGGTCAGCTTGATGCGAAAGAGATT
B.subtilis 168          AGGAAGAAAAATATGTGTGGAATCGTAGGTTATATCGGTCAGCTTGATGCGAAGGAAATT
                        ********** ********** ******** ********************* ** ***

B.subtilis TISTR25      TTGTTAAAAGGATTAGAGAAGCTTGAGTACCGCGGTTATGACTCTGCCGGTATCGCTGTG
B.subtilis 168          TTATTAAAAGGGTTAGAGAAGCTTGAGTATCGCGGTTATGACTCTGCTGGTATTGCTGTT
                        ** ******** ********** ********** ***** *****

B.subtilis TISTR25      GCGAATGAGCAGGGCGTGCATGTGTACAAAGAAAAAGGCCGCATCGCCGACCTTCGTGAA
B.subtilis 168          GCCAACGAACAGGGAATCCATGTGTTCAAAGAAAAAGGACGCATTGCAGATCTTCGTGAA
                        ** ** ** *****  * ******* ********** ***** ** ** *********

B.subtilis TISTR25      GTGGTGGATCACACGGTTGAATCTCAAGCGGGAATCGGCCATACACGCTGGGCGACTCAC
B.subtilis 168          GTTGTGGATGCCAATGTAGAAGCGAAAGCCGGAATTGGGCATACTCGCTGGGCGACACAC
                        ** ******  **  ** *** *  **** ***** ** ***** ********** ***

B.subtilis TISTR25      GGTGAACCAAGCTTCCTGAACGCTCACCCGCATCAAAGCGCACTCGGCCGCTTTACACTT
B.subtilis 168          GGCGAACCAAGCTATCTGAACGCTCACCCGCATCAAAGCGCACTGGGCCGCTTTACACTT
                        ** **********  ********************************* ***************

B.subtilis TISTR25      GTTCACAATGGTGTGATCGAGAACTATGTTCAGCTGAAGCGCGAATATCTTGAAAACGTT
B.subtilis 168          GTTCACAACGGCGTGATCGAGAACTATGTTCAGCTGAAGCAAGAGTATTTGCAAGATGTA
                        ******** ** ****************************  ** *** *  ** * **

B.subtilis TISTR25      GAACTGAAAAGCGACACGGACACTGAAGTAGTCGTTCAAATGATCGAGCAATTTGTGGCG
B.subtilis 168          GAGCTCAAAAGTGACACCGATACAGAAGTAGTCGTTCAAGTAATCGAGCAATTCGTCAAT
                        ** ** ***** ***** ** ** ********** * ********** **

B.subtilis TISTR25      GGAGGACTCAGCACAGAAGAAGCGTTCCGCAAAACACTGACTCTGTTAAAAGGCTCTTAC
B.subtilis 168          GGAGGACTTGAGACAGAAGAAGCGTTCCGCAAAACACTTACACTGTTAAAAGGCTCTTAT
                        ********    ************************** ** ****************

B.subtilis TISTR25      GCAATTGCATTATTTGACGGTGAAAACACAGACACCATTTACGTTGCAAAAAACAAAAGC
B.subtilis 168          GCAATTGCTTTATTCGATAACGACAACAGAGAAACGATTTTTGTAGCGAAAAACAAAAGC
                        ******** ***** **     ** **** *** ** ****  ** ** ************

B.subtilis TISTR25      CCTCTGTTAATCGGCCTTGGAGATACGTTTAACGTCGTGGCATCTGACGCGATGGCTATG
B.subtilis 168          CCTCTATTAGTAGGTCTTGGAGATACATTCAACGTCGTAGCATCTGATGCGATGGCGATG
                        ***** *** * ** ********** ** ******** ******** ******** ***

B.subtilis TISTR25      CTTCAAGTAACGAATGAATACGTTGAGCTTTTGGACAAAGAAATGGTGATCGTGACAAAA
B.subtilis 168          CTTCAAGTAACCAACGAATACGTAGAGCTGATGGATAAAGAAATGGTTATCGTCACTGAT
                        ********** ** ******** *****  **** ********** ***** **  *

B.subtilis TISTR25      GATGAAGCCGTGATTAAAAACCTTGACGGTGAAGTCATGACACGTGCGTCTTATATCGCT
B.subtilis 168          GACCAAGTTGTCATCAAAAACCTTGATGGTGACGTGATTACACGTGCGTCTTATATTGCT
                        **  ***  ** ** ********** ***** ** ** ****************** ***
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Figure 3.13 Sequence alignment of *B. subtilis* TISTR25 and *B. subtilis* 168 *gcaA* gene by using ClustalX (1.64b). Asterisk (*) indicate identity.

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B.subtilis TISTR25      GAGCTTGACGCCAGTGATATCGAAAAAGGCACATACCCTCACTACATGTTAAAAGAAACG
B.subtilis 168          GAGCTTGATGCCAGTGATATCGAAAAAGGCACGTACCCTCACTACATGTTGAAAGAAACG
                        ******** ********************** ***************** *********

B.subtilis TISTR25      GATGAGCAGCCGCTTGTTATGCGCAAAATCATCCAAACGTATCAGGACGAAAACGGCAGA
B.subtilis 168          GATGAGCAGCCTGTTGTTATGCGCAAAATCATCCAAACGTATCAAGATGAAAACGGCAAG
                        ***********  ********************************** ** **********

B.subtilis TISTR25      CTGGCCGTGGCCGGCGATGTCGCTGACGCCGTGGCGGAAGCGGACCGCATTTATATCGTG
B.subtilis 168          CTGTCTGTGCCTGGCGATATCGCTGCCGCTGTAGCGGAAGCGGACCGCATCTATATCATT
                        *** * *** * ******* ****** *** ** ****************** ****** *

B.subtilis TISTR25      GCTTGCGGAACGAGCTACCACGCCGGTCTTGTCGGGAAACAATATATTGAAATGTGGGCA
B.subtilis 168          GGCTGCGGAACAAGCTACCATGCAGGACTTGTCGGTAAACAATATATTGAAATGTGGGCA
                        *  ******** ******** ** ** ******** ************************

B.subtilis TISTR25      AACGTACCGGTTGAAGTGCATGTAGCGAGTGAATTCTCTTACAACATGCCGCTTCTGTCT
B.subtilis 168          AACGTGCCGGTTGAAGTGCATGTAGCGAGTGAATTCTCCTACAACATGCCGCTTCTGTCT
                        ***** ********************************** **********************

B.subtilis TISTR25      AAGAAGCCGCTCTTTATCTTCCTTTCTCAAAGCGGAGAAACAGCGGACAGCCGCGCCGTG
B.subtilis 168          AAGAAACCGCTCTTCATTTTCCTTTCTCAAAGCGGAGAAACAGCAGACAGCCGCGCGGTA
                        ***** ******** ** ***************************** *********** **

B.subtilis TISTR25      CTTGTTCAAGTCAAAGCGCTGGGTCACAAAGCGCTGACGATTACAAACGTTCCGGGATCA
B.subtilis 168          CTCGTTCAAGTCAAAGCGCTCGGACACAAAGCCCTGACAATCACAAACGTACCTGGATCA
                        ** ****************** ** ******** ***** ** ******** ** ******

B.subtilis TISTR25      ACGCTTTCCCGTGAAGCGGATTACACATTGCTTCTGCACGCAGGCCCTGAGATCGCCGTG
B.subtilis 168          ACGCTTTCTCGTGAAGCTGACTATACATTGCTGCTTCATGCAGGCCCTGAGATCGCTGTT
                        ******** ******** ** ** ******** ** ** ****************** **

B.subtilis TISTR25      GCATCAACAAAAGCGTATACGGCTCAGATTGCCGTCCTCGCGATCCTTGCGTCCGTTGCA
B.subtilis 168          GCGTCAACGAAAGCATACACTGCACAAATCGCAGTTCTGGCGGTTCTTGCTTCTGTGGCT
                        ** ***** ***** ** ** ** ** ** ** ** ** *** * ***** ** ** **

B.subtilis TISTR25      GCAGAACGCAACGGCGTTGATATCGGTTTTGATTTAGTCAAAGAATTAGGTATCGCGGCA
B.subtilis 168          GCTGACAAAAATGGCATCAATATCGGATTTGACCTCGTCAAAGAACTCGGTATCGCTGCA
                        ** **    ** *** *  ******* *****  * ********* * ******** ***

B.subtilis TISTR25      AACGCCATGGAAGCCCTCTGCGACCAGAAGGACGAAATGGAAATGATCGCACGTGAGTAC
B.subtilis 168          AACGCAATGGAAGCTCTATGCGACCAGAAAGACGAAATGGAAATGATCGCTCGTGAATAC
                        ***** ******** ** ************ ******************** ***** ***

B.subtilis TISTR25      CTGACTGTTTCAAGAAACGCTTTCTTCATCGGCCGCGGCCTTGACTACTTCGTGTGTGTC
B.subtilis 168          CTGACTGTATCCAGAAATGCTTTCTTCATCGGACGCGGCCTTGACTACTTCGTATGTGTC
                        ******** ** ***** ************** ********************** ******

B.subtilis TISTR25      GAAGGCGCCCTGAAGCTGAAAGAGATTTCTTACATCCAGGCGGAAGGCTTCGCCGGCGGC
B.subtilis 168          GAAGGCGCACTGAAGCTGAAAGAGATTTCTTACATCCAGGCAGAAGGTTTTGCCGGCGGT
                        ******** ********************************** ***** ** ********

B.subtilis TISTR25      GAGCTGAAGCATGGAACAATCGCTCTGATTGAAGAAGGAACACCGGTCTTTGCGCTTGCG
B.subtilis 168          GAGCTAAAGCACGGAACGATTGCCTTGATCGAACAAGGAACACCAGTATTCGCACTGGCA
                        ***** ***** ***** ** **  **** *** ********** ** ** ** ** **

B.subtilis TISTR25      ACACAAGAACACGTCAACCTGAGCATCCGCGGTAATGTGAAGGAAGTCGCAGCCCGCGGC
B.subtilis 168          ACTCAAGAGCATGTAAACCTAAGCATCCGCGGAAACGTCAAAGAAGTTGCTGCTCGCGGA
                        ** ***** ** ** ***** ********** ** ** ** ***** ** ** *****

B.subtilis TISTR25      GCCAACACTTGCATCATCTCGCTGAAAGGCTTAGAAGACGCAGACGACAGATTCATCCTG
B.subtilis 168          GCAAACACATGCATCATCTCACTGAAAGGCCTAGACGATGCGGATGACAGATTCGTATTG
                        ** ***** ********** ********* **** ** ** ** ********* *  **

B.subtilis TISTR25      CCGGAAGTCAACCCTGCGCTTCGTCCGCTGGTTTCTGTTGTGCCATTGCAGCTGATCGCT
B.subtilis 168          CCGGAAGTAAACCCAGCGCTTGCTCCGTTGGTATCTGTTGTTCCATTGCAGCTGATCGCT
                        ******** ***** ******  **** **** ******** ******************

B.subtilis TISTR25      TACTACGCTGCACTGCACCGCGGCTGTGACGTTGATAAACCGCGCAACCTTGCGAAGAGT
B.subtilis 168          TACTATGCTGCACTGCATCGCGGCTGTGATGTGGATAAACCTCGTAACCTTGCGAAGAGT
                        ***** ********** ********** ** ******** ** ****************
```

Figure 3.13 (continued)

```
B.subtilis TISTR25      GTTACGGTGGAATAATATAT--TTAACCCCTTTGGTTA---TTA-------GTGTGAATT
B.subtilis 168          GTTACTGTGGAGTAATAAATGTTTAACCCCTTTGGATAAGATTATCTAAAGGGGTGTTTT
                        ***** ***** ***** ** ************** **   ***       * ***  **

B.subtilis TISTR25     TAA----AATAAAGTCGCGATGTTTATA----AAAAA--GATGC--GATGTTTAAAATAA
B.subtilis 168          TATGTCCAAAAGCGAAAGAACGTCTACAGTGGAAAAATGGATTAAAGAAGTTGATGGTAC
                        **     ** *  *     * ** ** *    *****  ***    ** *** *   **

B.subtilis TISTR25     AG----------TCG--CGANGTTTATAAAAAAGTTGCGAAGCTTACCCCTTTGGATATG
B.subtilis 168          AGGACCCGACTATCGATCGACCATGGCTATAGATTTAAGATG-TTCCTCATTAGATCGG
                        **          ***  ***   *    * * * **  ** * ** ** * ** ***  *

B.subtilis TISTR25     ATTATC---TAAAGGGGTGTT-----TTTGTGTCGAAAAGAAAAAGAACATCTAAAATTG
B.subtilis 168          TCAATCAGGTTAAAAGGCATTAAAACTTGGAGAGAAACTGCTTTTGCCTAACTGAGTTTT
                           ***   * **  **  **    ** * *   **  *     *   * ** *  **

B.subtilis TISTR25     ATAAGTGGATTAAAGAGGGTCGAG-GAACTGGC--AGTGGGGCATGATTATCAAGATCTT
B.subtilis 168          CTAATGTTAATACAGATATAAGAGAGCAGTTCCTCATTAAGATACGCTTTTGATAGCAGT
                         ***    * ** ***    *** * * *  *  * * *  *  * * ** * *      *

B.subtilis TISTR25     TCCTCATTAGGTCGGTCAACAAGATTAAAAGGTATAAAA-ACCGGCA-GACAACATGAGT
B.subtilis 168          TGGAATTTAGCATGCTAGAAATCCGAAAACCGGAGAGCCTATTGGAATGATAACGTGTTT
                        *     ****   * *  * *     ***  * * *    *  ** * ** *** **  *

B.subtilis TISTR25     TTTTATCGG--ATTTGGAACGAAACTACTTTTATTTAACTGAATTTTCTGATGTTATTTT
B.subtilis 168          TTTTATTGACAATTAATAAAGGGCAAAGAATTCTAGAAGTCGCACTTACAATGTACTTGT
                        ****** *   ***   ** *     *   ** *  ** *     **   ****  ** *

B.subtilis TISTR25     AGATATTCGTGAACAATTTCCTTTATTACCACAAGAAGAGACGTTTGCCATTGCTG----
B.subtilis 168          GGGATTAGGTTTTAAAGTTCATTCTTTTTTTATTGAGTACATTCCGGTTAAAGTTGAAAT
                         *   *  **    ** *** **  **       **  * *     *  *  * **

B.subtilis TISTR25     ---
B.subtilis 168          GAT
```

Figure 3.13 (continued)

Table 3.1 Amino acid composition of *B. subtilis* TISTR25 *gcaA* protein in comparison with *B. subtilis* 168 *gcaA* protein.

Amino acids	*B. subtilis* TISTR25 *gcaA* protein				*B. subtilis* 168 *gcaA* protein			
	n	n(%)	MW	MW(%)	n	n(%)	MW	MW(%)
A ala alanine	67	11.2	4759	7.3	66	11.0	4688	7.2
C cys cysteine	6	1.0	618	0.9	6	1.0	618	0.9
D asp aspartic acid	32	5.3	3680	5.6	35	5.8	4025	6.2
E glu glutamic acid	51	8.5	6581	10.1	46	7.7	5935	9.1
F phe phenylalanine	16	2.7	2353	3.6	17	2.8	2500	3.8
G gly glycine	45	7.5	2565	3.9	45	7.5	2565	3.9
H his histidine	15	2.5	2055	3.1	14	2.3	1918	2.9
I ile isoleucine	38	6.3	4297	6.6	40	6.7	4523	6.9
K lys lysine	32	5.3	4099	6.3	34	5.7	4355	6.7
L leu leucine	62	10.3	7011	10.7	60	10.0	6785	10.4
M met methionine	13	2.2	1703	2.6	12	2.0	1572	2.4
N asn asparagine	22	3.7	2508	3.8	25	4.2	2851	4.4
P pro proline	16	2.7	1552	2.4	17	2.8	1649	2.5
Q gln glutamine	19	3.2	2433	3.7	22	3.7	2817	4.3
R arg arginine	23	3.8	3590	5.5	20	3.3	3122	4.8
S ser serine	28	4.7	2436	3.7	27	4.5	2349	3.6
T thr threonine	32	5.3	3233	4.9	30	5.0	3031	4.6
V val valine	57	9.5	5646	8.6	59	9.8	5845	9.0
W trp tryptophan	2	0.3	372	0.6	2	0.3	372	0.6
Y tyr tyrosine	24	4.0	3913	6.0	23	3.8	3750	5.7
Total	600	100	65431	100	600	100	65296	100

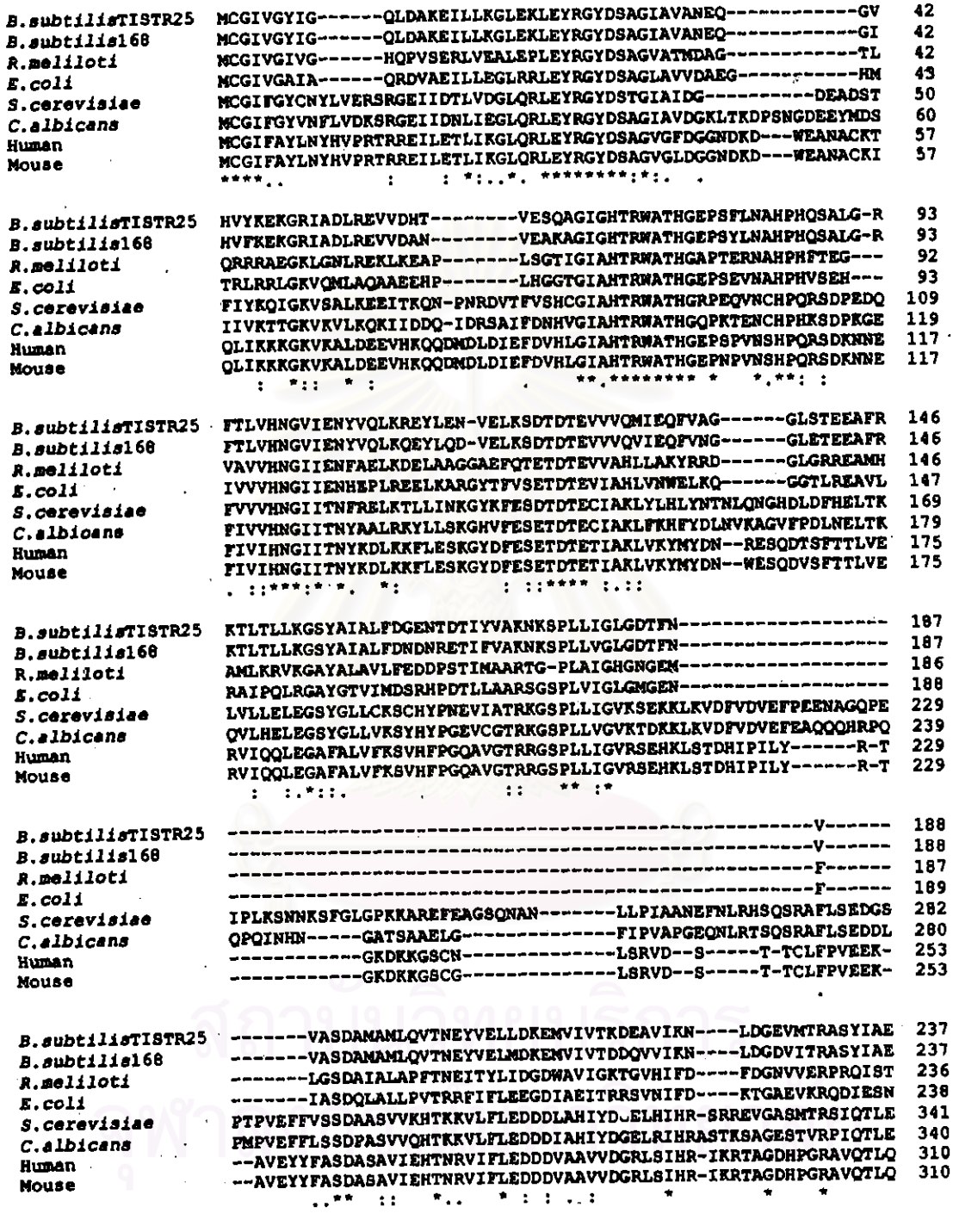


Figure 3.14 Alignment of the predicted amino acid sequence of *B. subtilis* TISTR25 *gcaA* open reading frame with those of 7 organisms. The amino acid sequences were aligned by using the ClustalX (1.64b) program. The numbers at the right-hand side are from initial methionine residue for each sequence. Gaps introduced to maximize this alignment are shown by dashes, identical residues are indicated by asterisks, and similar residues are indicated by dots.

```
B.subtilisTISTR25  LDASDIEKGTYPHYMLKETDEQPLVMRKIIQTYQD--ENGRLAVAGDVADA---VAEADR  292
B.subtilis168      LDASDIEKGTYPHYMLKETDEQPVVMRKIIQTYQD--ENGKLSVPGDIAAA---VAEADR  292
R.meliloti         AAAFLVDKGNHRHFMEKEIYEQPEVIAIALGHYVN---VIDKSCRSDSDAID--FAGVES  291
E.coli             LQYDAGDKGIYRHYMQKEIYEQPNAIKNTLTGRISH-GQVDLSELGPNADEL--LSKVEH  295
S.cerevisiae       MELAQIMKGPYDHFMQKEIYEQPESTFNTNRGRID--YENNKVILGGLKAWLPVVRRARR  399
C.albicans         MELNEIMKGPYKHFMQKEIFEQPDSAFNTNRGRID--FENCVVTLGGLKSWLSTIRRCRR  398
Human              MELQQIMKGNFSSFMQKEIFEQPESVVNTNRGRVN--FDDYTVNLGGLKDHIKEIQRCRR  368
Mouse              MELQQIMKGNFSSFMQKEIFEQPESVVNTNRGRVN--FDDYTVNLGGLKDHIKEIQRCRR  368
                          ** .  :* **  ***      :   .       .       .

B.subtilisTISTR25  IYIVACGTSYHAGLVGKQYIEMWANVPVEVHVASEFSYNMPLLSKKPLFIFLSQSGETAD  352
B.subtilis168      IYIIGCGTSYHAGLVGKQYIEMWANVPVEVHVASEFSYNMPLLSKKPLFIFLSQSGETAD  352
R.meliloti         LAISCCGTAYLAGLIGKYWFERYARLPVEIAVASEFRYREIPLSPQS-ALFISQSGETAD  350
E.coli             IQILACGTSYNSGMVSRYWFESLAGIPCDVEIASEFRYRKSAVRRNSLMITLSQSGETAD  355
S.cerevisiae       LIMIACGTSYHSCLATRAIFEELSDIPVSVELASDFLDRKCPVFRDDVCVFVSHCGETAD  459
C.albicans         IIMIACGTSYHSCLATRSIFEELTEIPVSVELASDFLDRRSPVFRDDTCVFVSQSGETAD  458
Human              LILIACGTSYHAGVATRQVLEELTELPVMVELASDFLDRNTPVFRDDVCFFLSQSGETAD  428
Mouse              LILIACGTSYHAGVATRQVLEELTELPVNVELASDFLDRNTPVFRDDVCFFISQSGETAD  428
                   : :  ***:* : :  :  :*  : :*  : :**:*  .   :  .   . :*:.*****

B.subtilisTISTR25  SRAVLVQVKALG-HKALTITNVPGSTLSREADYTLLLHAGPEIAVASTKAYTAQIAVLAI  411
B.subtilis168      SRAVLVQVKALG-HKALTITNVPGSTLSREADYTLLLHAGPEIAVASTKAYTAQIAVLAV  411
R.meliloti         TLASLRYCKAHG-LRIGAVVNARESTMARESDAVFPILAGPEIGVARTKAFTCQLAVLAA  409
E.coli             TLAGLRLSKELGYLGSLAICNVPGSSLVRESDLALNTNAGTEIGVASTKAFTTQLTVLLM  415
S.cerevisiae       TMLALNYCLERG-ALTVGIVNSVGSSISRVTHCGVHINAGPEIGVASTKAYTSQYIALVM  518
C.albicans         SILALQYCLERG-ALTVGIVNSVGSSMSRQTHCGVHINAGPEIGVASTKAYTSQYIALVM  517
Human              TLMGLRYCKERG-ALTVGITNTVGSSISRETDCGVHINAGPEIGVASTKAYTSQFVSLVM  587
Mouse              TLMGLRYCKERG-ALTVGITNTVGSSISRETDCGVHINAGPEIGVASTKAYTSQFVSLVM  587
                   :   *      *      : *   *:: * :.  .   **.**.** ***:* *   *

B.subtilisTISTR25  LASVAAERNG---VDIGFDLVKELGIAANAMEALCDQKD-EMEMIAREYLTVSRNAFFIG  467
B.subtilis168      LASVAADKNG---INIGFDLVKELGIAANAMEALCDQKD-EMEMIAREYLTVSRNAFFIG  467
R.meliloti         LRAGAGKARGTISGDEEQALIKSLAEMPAIMGQVLNSIQPEIEVLSRELSNC-RDVLYLG  468
E.coli             LVANVSRLKG-LDASIEHDIVHGLQALPSRIEQML-SQDKRIEALAEDFSDK-HHALFLG  472
S.cerevisiae       FALSLSDDRVS-KIDR-IEIIQGLKLIPGQIKQVLKLEP-RIKKLCATELKDQKSLLLLG  575
C.albicans         FALSLSNDSIS-RKGRHEEIIKGLQKIPEQIKQVLKLEN-KIKDLCNSSLNDQKSLLLLG  575
Human              FALMMCDDRIS-MQERRKEIMLGLKRLPDLIKEVLSMDD-EIQKLA-TELYHQKSVLIMG  544
Mouse              FALMMCDDRIS-MQERRKEIMLGLKRLPDLIKEVLSMDD-EIQKLA-TELYHQKSVLIMG  544
                   :                    ::  *   .  :  :      .:: :.       :  : :*

B.subtilisTISTR25  RGLDYFVCVEGALKLKEISYIQAEGFAGGELKHGTIALIEEGTPVFALATQEHVNLSIRG  527
B.subtilis168      RGLDYFVCVEGALKLKEISYIQAEGFAGGELKHGTIALIEQGTPVFALATQEHVNLSIRG  527
R.meliloti         RGTSFPLAMEGALKLKEISYIQPKSYAAGQLKHGPYALIDENMPVIVIAPHDRFFDKTVT  528
E.coli             RGDQYPIALEGALKLKEISYIHAEAYAAGELKHGPLALIDADMPVIVVAPNNELLEKLKS  532
S.cerevisiae       RGYQFAAALEGALKIKEISYMHSEGVLAGELKHGVLALVDENLPIIAFGTRDSLFPKVVS  635
C.albicans         RGYQFATALEGALKIKEISYMHSEGVLAGELKHGILALVDEDLPIIAFATRDSLFPKVMS  635
Human              RGYHYATCLEGALKIKEITYMHSEGILAGELKHGPLALVDKLMPVIMIIMRDHTYAKCQN  604
Mouse              RGYHYATCLEGALKIKEITYMHSEGILAGELKHGPLALVDKLMPVIMIIMRDHTYAKCQN  604
                   **  :  .:*****:***:*::.:.  .*:****  **::   *:: .  .:    .

B.subtilisTISTR25  NVKEVAARGANTCIIS--------LKGLEDADDRFILPEVNPALRPLVSVVPLQLIAYYA  579
B.subtilis168      NVKEVAARGANTCIIS--------LKGLDDADDRFVLPEVNPALAPLVSVVPLQLIAYYA  579
R.meliloti         NMQEV-ARGGRIILITDEKG---AAASKLDTMHTIVLPEVDEIIAPMIFSLPLQLLAYHT  584
E.coli             NIEEVRARGGQLYVFADQDA----GFVSSDNMHIIEMPHVEEVIAPIFYTVPLQLLAYHV  588
S.cerevisiae       SIEQVTARKGHPIIICNENDEVWAQKSKSIDLQTLEVPQTVDCLQGLINIIPLQLMSYWL  695
C.albicans         AIEQVTARDGRPIVICNEGD---AIISNDKVHTTLEVPETVDCLQGLLNVIPLQLISYWL  692
Human              ALQQVVARQGRPVVICDKED----TETIKNTKRTIKVPHSVDCLQGILSVIPLQLLAFHL  660
Mouse              ALQQVVARQGRPVVICDKED----TETIKNTKRTIKVPHSVDCLQGILSVIPLQLLAFHL  660
                    :::* ** ..  ::                    : :*.    :  :.  :****:::

B.subtilisTISTR25  ALHRGCDVDKPRNLAKSVTVE  600
B.subtilis168      ALHRGCDVDKPRNLAKSVTVE  600
R.meliloti         AVFMGTDVDQPRNLAKSVTVE  605
E.coli             ALIKGTDVDQPRNLAKSVTVE  609
S.cerevisiae       AVNKGIDVDFPRNLAKSVTVE  716
C.albicans         AVNRGIDVDFPRNLAKSVTVE  713
Human              AVLRGYDVDFPRNLAKSVTVE  681
Mouse              AVLRGYDVDFPRNLAKSVTVE  681
                   *:  * *** **********
```

Figure 3.14 (continued)

Table 3.2 Estimated genetic distances among nucleotide sequences of the *gcaA* gene from 8 organisms

	Human	Mouse	*S.cerevisiae*	*C.albicans*	*B.subtilis* TISTR25	*B.subtilis* 168	*R.meliloti*	*E. coli*
Human	-							
Mouse	0.0972	-						
S. cerevisiae	0.6511	0.6800	-					
C. albicans	0.6506	0.6837	0.3666	-				
B. subtilis TISTR25	1.1735	1.2018	1.1549	1.1987	-			
B. subtilis 168	1.1185	1.1457	1.1091	1.0688	0.1705	-		
R. meliloti	1.2731	1.2144	1.1958	1.3233	0.9000	0.9302	-	
E. coli	1.0678	1.0592	1.0764	1.1147	1.0080	1.0579	0.8679	-

Table 3.3 Estimated genetic distances among amino acid sequences of the *gcaA* protein from 8 organisms

	Human	Mouse	*S.cerevisiae*	*C.albicans*	*B.subtilis* TISTR25	*B.subtilis* 168	*R.meliloti*	*E. coli*
Human	-							
Mouse	0.01002	-						
S. cerevisiae	0.63101	0.63548	-					
C. albicans	0.63200	0.63106	0.33108	-				
B. subtilis TISTR25	1.38190	1.38313	1.36734	1.37984	-			
B. subtilis 168	1.38757	1.38449	1.36790	1.36973	0.06940	-		
R. meliloti	1.32260	1.31277	1.31340	1.34859	1.10600	1.07633	-	
E. coli	1.12711	1.13071	1.15064	1.13109	1.09629	1.08505	0.87441	-

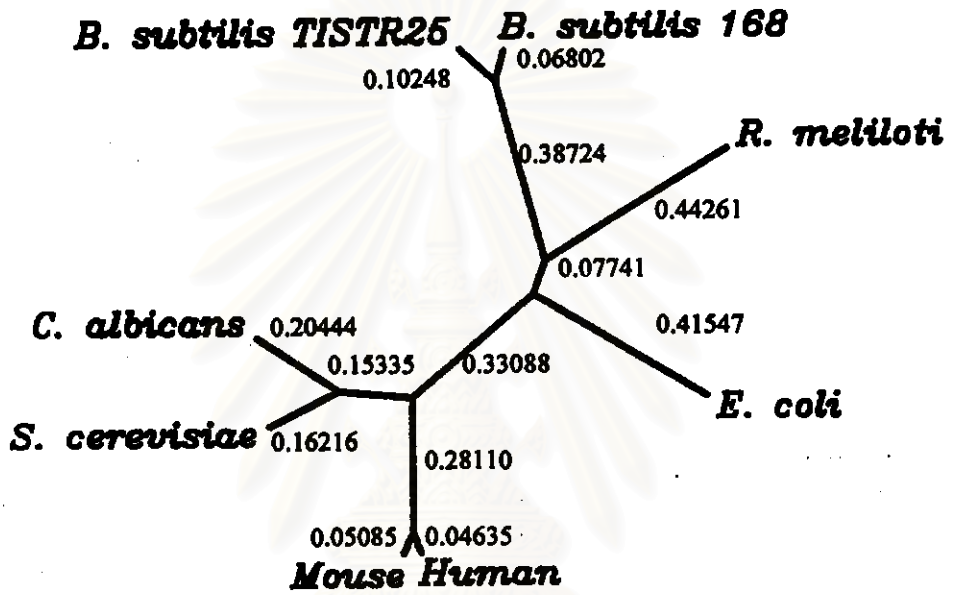


Figure 3.15 A phylogenetic tree of the *gcaA* gene from *B. subtilis* TISTR25 and 7 organisms based on nucleotide sequence divergences

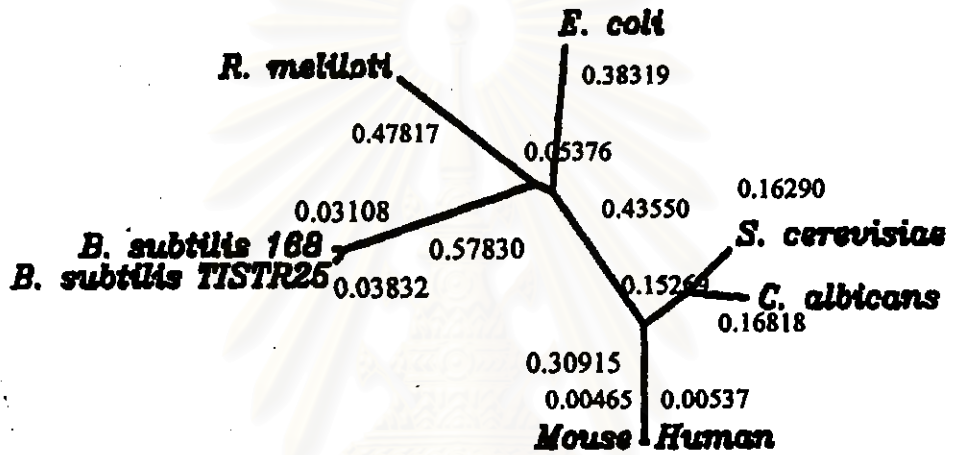
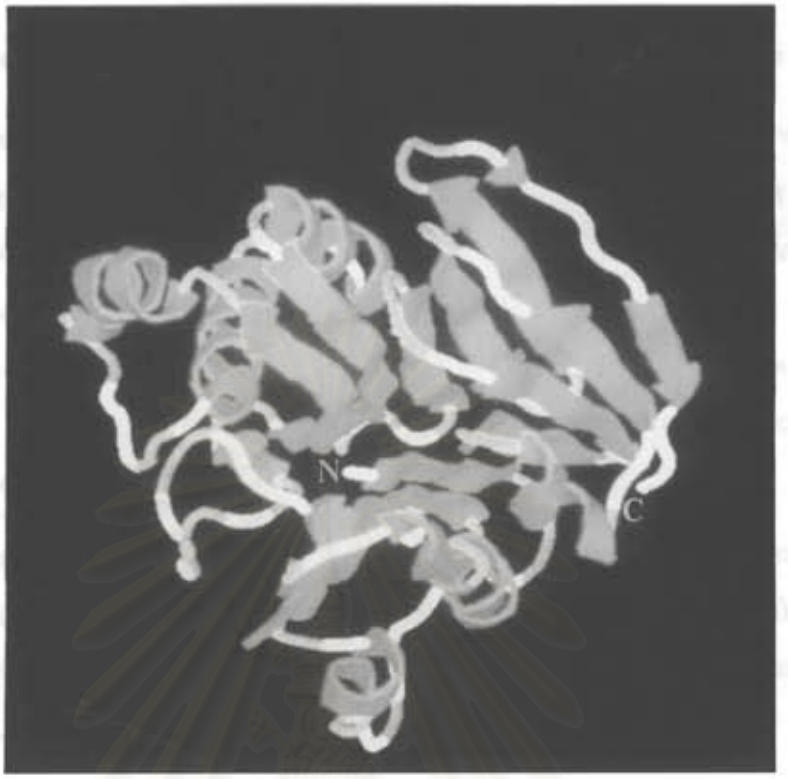
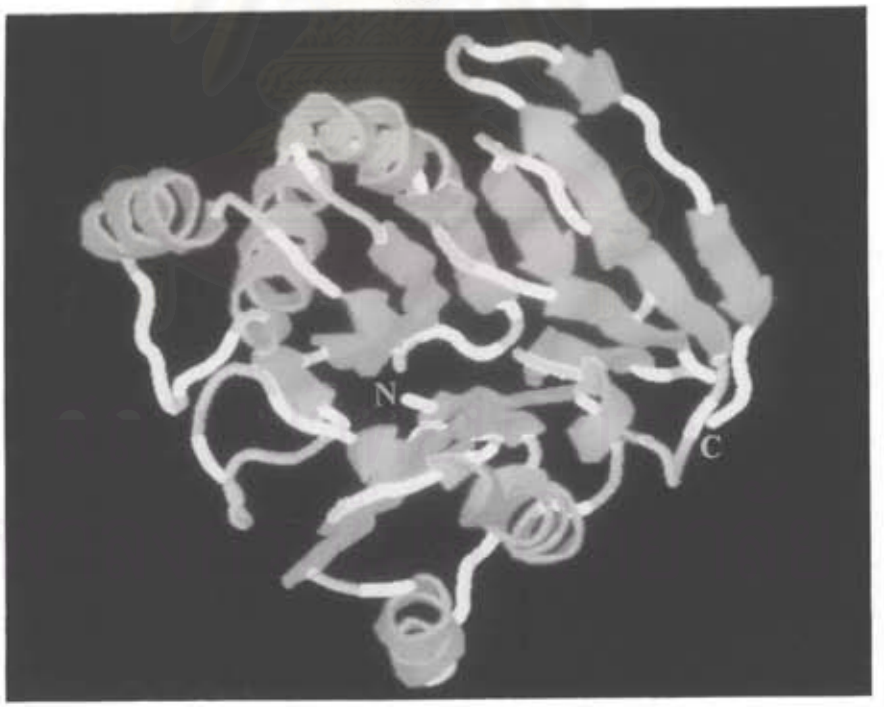


Figure 3.16 A phylogenetic tree of the *gcaA* protein from *B. subtilis* TISTR25 and 7 organisms based on amino acid sequence divergences



(a)



(b)

Figure 3.17 The predicted three-dimensional structure of the *gcaA* protein
(a) *B. subtilis* TISTR25 *gcaA* protein (237 amino acids)
(b) *E. coli gcaA* protein in chain A (238 amino acids)

3.4 Assay of L-glutamine D-fructose-6-phosphate amidotransferase activity

Sequences analysis clearly showed that pCSBC14 insert fragment was a *gcaA* gene. This clone was then assayed to clarify whether it had *gcaA* protein activity or not. To test whether or not the *gcaA* gene was under the influence of *lac* promoter, IPTG was also included in the culture. *E. coli* DH5α harboring either pUC18 or pCSBC14 were cultivated in the presence or absence of IPTG. The cell extracts were prepared for the assay of L-glutamine D-fructose-6-phosphate amidotransferase activity as described in section 2.11.

The result of enzyme assay were shown in Table 3.4. The specific activity of *gcaA* protein of pUC18 was 96% less than that of pCSBC14. The specific activities of *gcaA* protein of pCSBC14 with and without IPTG were not significant different (36.01 and 36.23 units/mg protein).

Table 3.4 L-glutamine D-fructose-6-phosphate amidotransferase activity in cell extracts.

E. coli DH5α harboring	Packed cells (g)	Crude extract (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
pUC18	1.15	4.4	23.60	34.25	1.45
pUC18+IPTG	1.18	4.5	21.60	28.69	1.33
pCSBC14	1.05	4.0	18.88	679.86	36.01
pCSBC14+IPTG	0.94	3.5	15.36	556.55	36.23

3.5 Southern-blot hybridization analysis of *B. subtilis* TISTR25 chromosomal DNA with neutral and alkaline protease probes

Since pCSBC14 was a clone of *gcaA* gene instead of the desired protease gene, attempt to clone protease gene was made. The protease gene from *B. subtilis* TISTR25 was then traced by Southern-blot hybridization using protease probes for both neutral and alkaline proteases.

3.5.1 Isolation of *B. subtilis* TISTR25 chromosomal DNA

The chromosomal DNA of *B. subtilis* TISTR25 was extracted by protocol described in section 2.13.1. The amount of DNA obtained was estimated by comparison of its intensity in a 0.7% agarose gel eletrophoresis with that of λ/*Hin*dIII marker (Figure 3.18). The DNA was shown as a high molecular weight DNA (>23.1 kb).

3.5.2 Digestion of *B. subtilis* TISTR25 chromosomal DNA

1 μg of *B. subtilis* TISTR25 chromosomal DNA was digested separately with 8 restriction endonucleases, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I, and run on 0.7% agarose gel along with λ/*Hin*dIII marker (Figure 3.19). The DNA fragments were transferred from agarose gel onto nylon membrane before hybridization with protease probes.

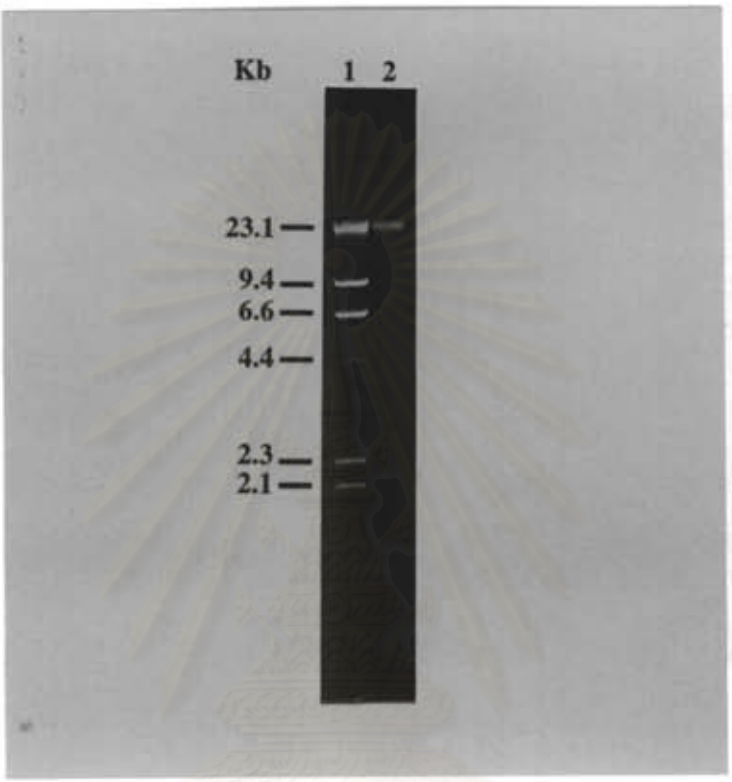


Figure 3.18 Chromosomal DNA of *B. subtilis* TISTR25

Lane 1 λ/*Hin*dIII

Lane 2 *B. subtilis* TISTR25 chromosomal DNA

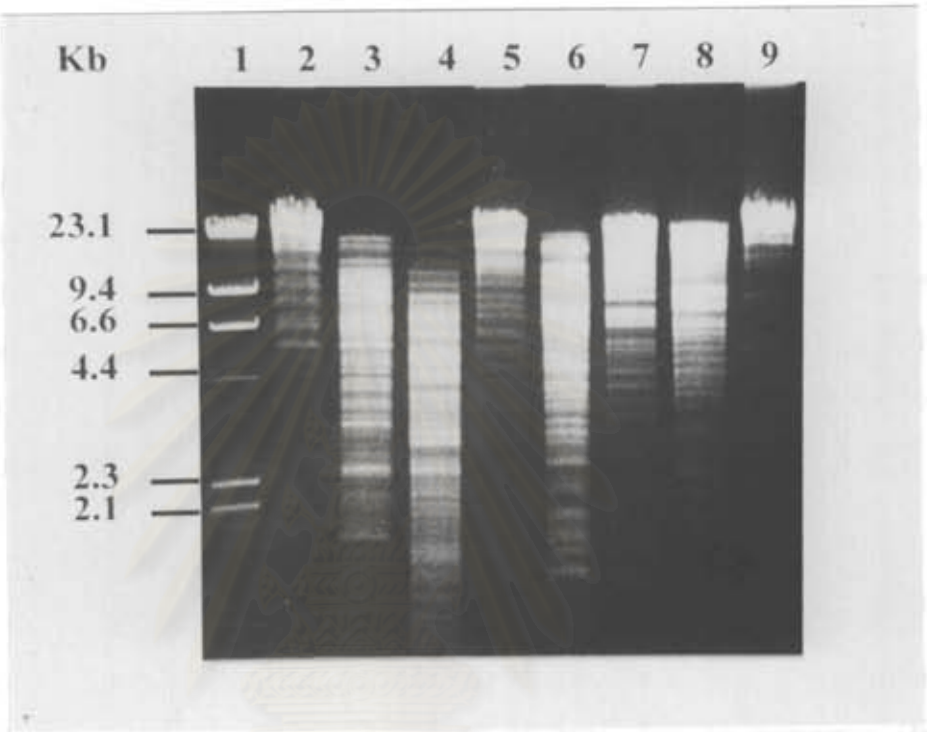


Figure 3.19 Digestion of *B. subtilis* TISTR25 chromosomal DNA with 8 restriction endonuclease

Lane 1	λ/*Hin*dIII
Lane 2	DNA digested with *Bam*HI
Lane 3	DNA digested with *Eco*RI
Lane 4	DNA digested with *Hin*dIII
Lane 5	DNA digested with *Kpn*I
Lane 6	DNA digested with *Pst*I
Lane 7	DNA digested with *Sal*I
Lane 8	DNA digested with *Sma*I
Lane 9	DNA digested with *Xba*I

3.5.3 Southern-blot hybridization analysis

Protease gene in the chromosomal DNA of *B. subtilis* TISTR25 was traced by using neutral and alkaline protease probes.

3.5.3.1 Neutral protease probe

The 600 bp DNA fragment of neutral protease, obtained from the digestion of pNC3 with *Eco*RI and *Bgl*I, was used as a probe. This probe was radioactively labelled by using nick translation. The results of hybridization showed that each of the *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Sma*I digested *B. subtilis* TISTR25 DNA gave one strong signal band (Figure 3.20), ranging in size from 2.5-12.0 kb (Table 3.5). This result implied that the neutral protease gene existed in the *B. subtilis* TISTR25 chromosomal DNA.

3.5.3.2 Alkaline protease probe

The alkaline protease probe, an oligonucleotide of 20 bases (5′-TGTCGCAAGCACCGCACTAC-3′) was designed from the nucleotide sequence of *Bacillus* alkaline protease gene and synthesized by automatic synthesizer. The oligonucleotide of alkaline protease probe was 5′end labelled with [γ-^{32}P]dATP. The result of hybridization showed that each of the digested DNA revealed more than one signal band (Figure 3.21).

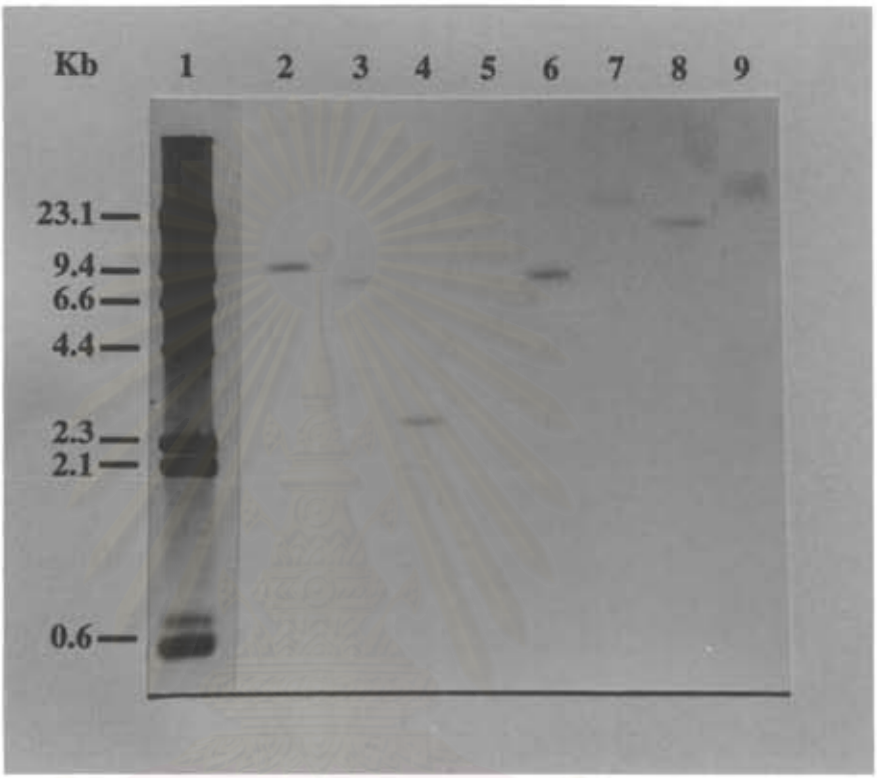


Figure 3.20 Southern-blot hybridization analysis of digested *B. subtilis* TISTR25 chromosomal DNA with neutral protease probe

Lane 1	λ/*Hin*dIII
Lane 2	DNA digested with *Bam*HI
Lane 3	DNA digested with *Eco*RI
Lane 4	DNA digested with *Hin*dIII
Lane 5	DNA digested with *Kpn*I
Lane 6	DNA digested with *Pst*I
Lane 7	DNA digested with *Sal*I
Lane 8	DNA digested with *Sma*I
Lane 9	DNA digested with *Xba*I

Table 3.5 An approximately size of digested *B. subtilis* TISTR25 fragment from the hybridization with neutral protease probe

Restriction enzyme digested *B. subtilis* TISTR25	Approximate size (kb)
*Bam*HI	7.8
*Eco*RI	7.2
*Hin*dIII	2.5
*Pst*I	6.9
*Sma*I	12.0

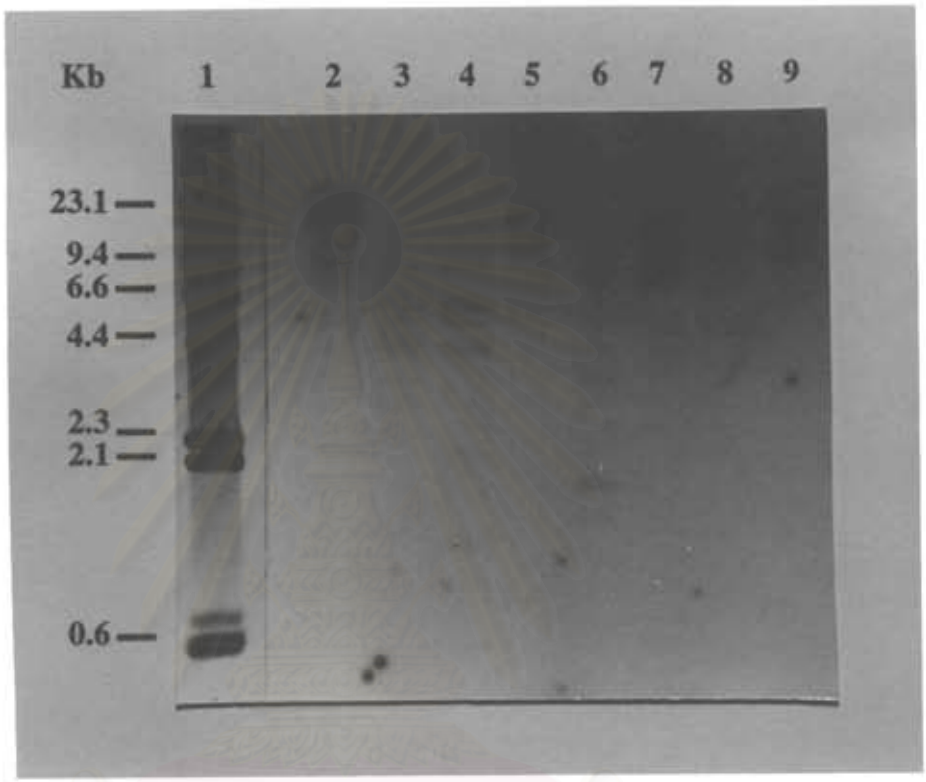


Figure 3.21 Southern-blot hybridization analysis of digested *B. subtilis* TISTR25 chromosomal DNA with alkaline protease probe

Lane 1	λ/*Hin*dIII
Lane 2	DNA digested with *Bam*HI
Lane 3	DNA digested with *Eco*RI
Lane 4	DNA digested with *Hin*dIII
Lane 5	DNA digested with *Kpn*I
Lane 6	DNA digested with *Pst*I
Lane 7	DNA digested with *Sal*I
Lane 8	DNA digested with *Sma*I
Lane 9	DNA digested with *Xba*I